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$p27^{Kip1}$ alters the response of cells to mitogen and is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation

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Background: In many vertebrate cell lineages, precursor cells divide a limited number of times before they arrest and terminally differentiate into postmitotic cells. It is not known what causes them to stop dividing. We have been studying the 'stopping' mechanism in the proliferating precursor cells that give rise to oligodendrocytes, the cells that make myelin in the central nervous system. We showed previously that the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (p27) progressively accumulates in cultured precursor cells as they proliferate and that the time course of the increase is consistent with the possibility that p27 accumulation is part of a cell-intrinsic timer that arrests the cell cycle and initiates differentiation at the appropriate time.

Results: We now provide direct evidence that p27 is part of the intrinsic timer. We show that although $p27^{-/-}$ precursor cells stop dividing and differentiate almost as fast as wild-type cells when deprived of mitogen, when stimulated by saturating amounts of mitogen they have a normal cell-cycle time but tend to go through one or two more divisions than wild-type cells before they stop and differentiate. Cells that are $p27^{+/-}$ behave in an intermediate way, going through at most one extra division, indicating that the levels of p27 matter in the way the timer works. We also show that $p27^{-/-}$ precursor cells are more sensitive than wild-type cells to the mitogenic effect of platelet-derived growth factor.

Conclusions: These findings demonstrate that p27 is part of the normal timer that determines when oligodendrocyte precursor cells stop dividing and differentiate, at least *in vitro*. It seems likely that p27 plays a similar role in many other cell lineages, which could explain the phenotypes of the $p27^{-/-}$ and $p27^{+/-}$ mice.

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Background

The cellular and molecular mechanisms that control the size of an animal or an organ are poorly understood, and, despite their fundamental importance, they have received surprisingly little attention. Size largely reflects cell numbers, which are determined mainly by controls on cell proliferation and cell death [1]. We have been studying these controls in the rodent optic nerve, focusing on the mechanisms that determine the final number of oligodendrocytes, the cells that myelinate the axons in the nerve [2]. The oligodendrocytes develop from dividing precursor cells that migrate into the developing optic nerve from the brain, beginning before birth [3]. The precursors divide a limited number of times before they stop and terminally differentiate into postmitotic oligodendrocytes [4], which first appear in the rat optic nerve around the day of birth, following which their numbers increase for 6 weeks [5].

Clonal analyses of either single [4] or purified [6] oligodendrocyte precursor cells suggest that a cell-intrinsic timer plays an important part in determining when a precursor cell stops dividing and differentiates. When precursor cells isolated from postnatal day 7–8 (P7–8) rat optic nerve, for example, are stimulated to proliferate in culture by either astrocytes or platelet-derived growth factor (PDGF), they divide from zero to eight times before they stop and differentiate, and the progeny of an individual precursor cell tend to stop dividing and differentiate at about the same time [4,6]. Moreover, if the two daughter cells of an individual precursor cell are separated and cultured on astrocyte monolayers in separate microwells, they tend to divide the same number of times before they differentiate, suggesting that a cell-intrinsic timing or counting mechanism limits the time or number of times that a precursor cell normally divides [4]. The finding that precursor cells cultured at 33°C divide more slowly but stop dividing and differentiate earlier, after fewer divisions, than at 37°C suggests that the mechanism does not operate by simply counting cell divisions but probably measures elapsed time in some other way [7].

For the intrinsic timer to operate normally at least two kinds of extracellular signaling molecules are required: the mitogen PDGF [8–10] and hydrophobic signals such as

thyroid hormone (TH) or retinoic acid [6]. The need for PDGF is indicated by the finding that single [4] or purified [6] precursor cells cultured in the absence of PDGF stop dividing and differentiate into oligodendrocytes within 1–2 days, whether or not hydrophobic signals are present [4,6]. The need for hydrophobic signals is indicated by the finding that most precursor cells cultured in the presence of PDGF but in the absence of hydrophobic signals tend to keep dividing without differentiating [6]. If TH is added to such cultures after 8 days, however, the precursor cells rapidly stop dividing and differentiate [6]. This finding and others [11] suggest that some kind of timing mechanism continues to operate even in the absence of hydrophobic signals and that the cell-intrinsic timer consists of at least two components — a timing component that measures elapsed time independently of hydrophobic signals, and an effector component that depends on hydrophobic signals and stops cell proliferation and initiates differentiation when the timing component indicates it is time to do so.

How might the intrinsic timer operate to stop cell division at the correct time? Whatever the mechanism, in the end it must interact with the intracellular control system that regulates progress through the cell-division cycle. In principle, the timer could depend on the decay of intracellular stimulatory proteins that normally drive progress through the cell cycle, the accumulation of intracellular inhibitory proteins that normally retard progress through the cycle, or both of these mechanisms. The eucaryotic cell cycle is controlled by a family of cyclin-dependent protein kinases (Cdks), which are cyclically activated to trigger the different phases of the cell cycle at the right time and in the right sequence [12–14]. These kinases are regulated by a variety of proteins, including the cyclins, which activate them, the kinases and phosphatases that activate or inhibit them [12,13], and the Cdk inhibitors, which block the assembly or activity of the cyclin–Cdk complexes [15]. Two families of Cdk inhibitors have been identified in mammalian cells — the Cip/Kip family, which includes $p27^{Kip1}$, $p21^{Cip1}$ and $p57^{Kip2}$, and the Ink4 family, which includes $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$ [15]. These inhibitors can inhibit the various cyclin–Cdk complexes that control G1 progression and entry into S phase, and their overexpression in various cell lines arrests the cell cycle in G1 [15]. Moreover, some of them are upregulated when developing cells exit from the cell cycle [16–18], raising the possibility that they play a role in stopping the cycle at the appropriate time.

We showed previously that in the presence of PDGF and the absence of TH, which are conditions where the counting component of the timer continues to operate normally but the effector component does not [6], the Cdk inhibitor $p27^{Kip1}$ (p27) progressively increases in oligodendrocyte precursor cells as they proliferate in culture [19]. The time course of this increase is consistent with the possibility that p27 accumulation is part of the timing component of the intrinsic timer. We also showed that in the absence of PDGF, p27 levels rise very rapidly and remain high after the cells stop dividing and differentiate into oligodendrocytes [19], raising the possibility that p27 may also be part of the effector component of the timer, although in this case cell-cycle arrest was triggered by PDGF deprivation rather then by the timing component. The accumulation of p27, however, was not sufficient on its own to arrest cell division and initiate differentiation in the presence of PDGF and the absence of TH, suggesting that p27 is only one component of the timer [19].

After we had completed these studies, three laboratories [20–22] independently reported that mice in which both copies of the p27 gene are inactivated by targeted gene disruption grow more rapidly and are about one third larger than normal, despite normal serum levels of growth hormone (GH) and insulin-like growth factor 1 (IGF-1). Interestingly, mice with only one p27 gene inactivated are about one sixth larger than normal. All of the organs examined in the $p27^{-/-}$ mice are increased in size and contain more cells than normal, apparently as a result of increased cell division rather than decreased cell death. There are at least three possible explanations for the $p27^{-/-}$ phenotype: firstly, p27 normally plays a part in limiting cell proliferation in many cell lineages; secondly, $p27^{-/-}$ cells are more sensitive to mitogens; or thirdly, some unknown neuroendocrine mechanism that regulates cell proliferation in many organs independently of serum GH and IGF-1 is perturbed in these mice.

In the present study we have compared the proliferative behavior of oligodendrocyte precursor cells isolated from the developing optic nerve of normal and p27-deficient mice. We show that many p27-deficient precursor cells cultured at clonal density in the presence of PDGF and TH go through one or two more divisions than wild-type precursors before they stop and differentiate, apparently as a result of cell-autonomous defects in both the timing and effector components of the timer in these cells. These findings demonstrate that p27 is part of the normal timer that determines when oligodendrocyte precursor cells stop dividing and terminally differentiate, at least *in vitro*. In addition, we show that the $p27^{-/-}$ precursors are more sensitive to the mitogenic effect of PDGF, suggesting that p27 normally regulates mitogen sensitivity in these cells. It seems likely that p27 plays similar roles in many cell lineages, providing a probable explanation for the generalized hyperplasia observed in $p27^{-/-}$ mice.

After these studies were completed, Casaccia-Bonnefil *et al.* [23] reported that most oligodendrocyte precursor cells isolated from cultures of neonatal $p27^{-/-}$ mouse brain fail

to stop dividing and differentiate when they are deprived of PDGF in culture. Their findings are significantly different from ours, and we provide a likely explanation for why this may be. Most importantly, Casaccia-Bonnefil *et al.* did not study the effect of the p27 deficiency on the intrinsic timer in the precursor cells.

Results

p27 genotyping

As female p27$^{-/-}$ mice are sterile [20–22], we mated p27$^{+/-}$ or p27$^{-/-}$ males with p27$^{+/-}$ females and assessed the genotype of each of the progeny by western blotting of extracts of brain using anti-p27 antibodies. As described previously [20], no p27 protein was detectable in p27$^{-/-}$ mice, and the level of p27 protein in p27$^{+/-}$ animals was about half the level of expression detected in p27$^{+/+}$ mice (data not shown). Optic nerve cells from each mouse pup were prepared, cultured and assessed separately.

Growing and identifying mouse oligodendrocyte lineage cells in culture

Our previous studies on the timing of oligodendrocyte development in culture made use of rat optic nerve cells, where clonal culture conditions [6] and markers for identifying the various cell types [24,25] have been well worked out. In the present study we found that these culture conditions and some of the markers were unsuitable for mouse optic nerve cells. For mouse oligodendrocyte lineage cells to survive and proliferate at low cell density in culture, for example, we had to increase the concentration of the adenylyl cyclase activator forskolin from 5 μM to 20 μM. For these cells to survive at clonal density, we had, in addition, to supplement the culture medium with either 50% (volume : volume) astrocyte-conditioned medium or 5% Müller-cell-conditioned medium (see Materials and methods), in which case the cells survived for at least 10 days.

As in rat cultures, we identified oligodendrocytes by their characteristic morphology and, in some cases, by their cell-surface expression of galactocerebroside (GC) [24,26]. The A2B5 monoclonal antibody [27], which can be used to identify oligodendrocyte precursor cells in cultures of rat optic nerve [25], cannot be used in this way for mouse optic nerve cells, as it labels many non-oligodendrocyte-lineage cells and does not label a substantial proportion of the oligodendrocyte precursor cells ([28] and our unpublished observations). Instead, we used a combination of cell morphology [4] and labeling with an antiserum against the NG2 proteoglycan [28,29] to identify the precursor cells. Although NG2 is also expressed by meningeal cells, these cells have a fibroblast-like morphology, which is easily distinguished from the process-bearing morphology of oligodendrocyte precursor cells. NG2 is rapidly lost as the precursor cells differentiate into GC^{+} oligodendrocytes ([28,29] and this study).

Number of precursor cell divisions in clonal-density culture

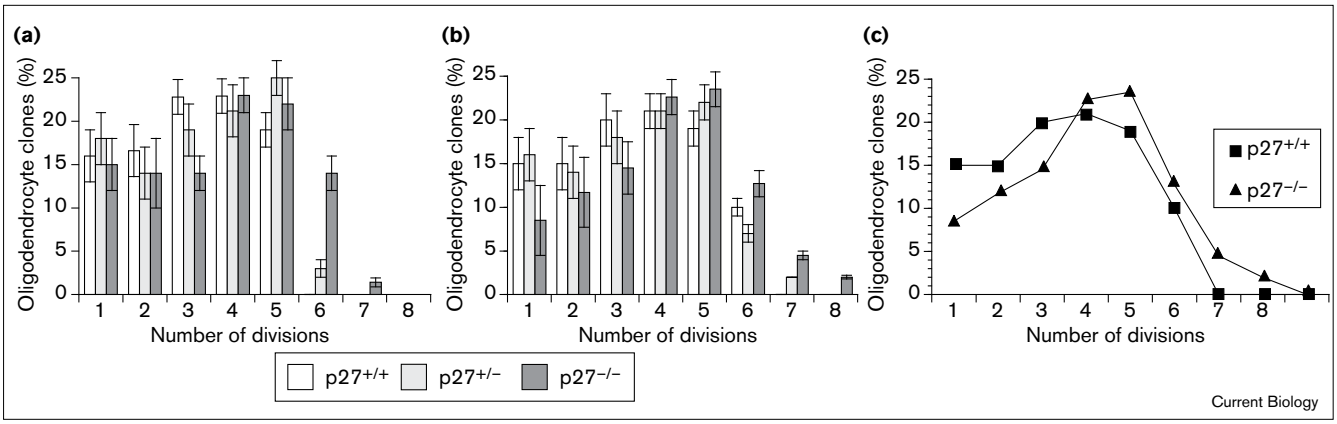
To determine if p27 influences the number of times that oligodendrocyte precursor cells divide before they stop and differentiate, we cultured P7 optic nerve cells from p27$^{+/+}$, p27$^{+/-}$ and p27$^{-/-}$ mice at clonal density in the presence of PDGF and TH and added fresh medium and PDGF every 2 days. After 5 and 7 days, we counted the number of cells in each oligodendrocyte clone, which we defined as a clone in which the majority of the cells had the morphology of typical oligodendrocytes. As there was little cell death in these cultures, and oligodendrocytes are produced in clonal cultures of P7 precursor cells by a proliferative lineage, in which symmetrical divisions generate daughters that tend to differentiate at around the same time [4,30], we converted the number of cells in each clone to the number of divisions that the precursors underwent before differentiating. We probably overestimated the number of small clones (having undergone one or two divisions), some of which may have represented clusters of two to four oligodendrocytes in the starting population. It has been shown previously [4,6] that P7–8 oligodendrocyte precursor cells are heterogeneous in their proliferative capacity, probably reflecting heterogeneity in their maturation [30]. As can be seen in Figure 1a, by 5 days *in vitro* none of the p27$^{+/+}$ oligodendrocyte clones had undergone more than five divisions, whereas some p27$^{+/-}$ clones had undergone six divisions, and some p27$^{-/-}$ clones had undergone seven divisions. At this time there were still many clones that contained mainly precursor cells, confirming that PDGF was not limiting in these cultures and that the observed oligodendrocyte differentiation reflected the operation of the intrinsic timer that limits the proliferation of the precursor cells in saturating concentrations of PDGF. By 7 days *in vitro*, almost all of the clones in cultures of all genotypes contained only oligodendrocytes, presumably because almost all of the precursor cells had now been triggered to differentiate by their intrinsic timer. None of the p27$^{+/+}$ clones had undergone more than six divisions, whereas some p27$^{+/-}$ clones had undergone seven divisions, and some p27$^{-/-}$ clones had undergone eight divisions (Figure 1b). Thus, in these culture conditions some p27-deficient precursor cells went through one to two divisions more than any wild-type precursor cells, and p27$^{-/-}$ cells tended to go through more divisions than p27$^{+/-}$ cells.

Although the increase in clonal size was most obvious in the largest p27$^{-/-}$ clones, it was also apparent in smaller clones. This can be seen in Figure 1c, where the data in Figure 1b are replotted as curves, and the p27$^{-/-}$ curve is shifted to the right compared to the wild-type curve. Thus, many p27$^{-/-}$ precursor cells go through one or two extra divisions in these conditions.

Cell-cycle time

The differences in proliferative behavior between the oligodendrocyte precursor cells from the three types of

Figure 1



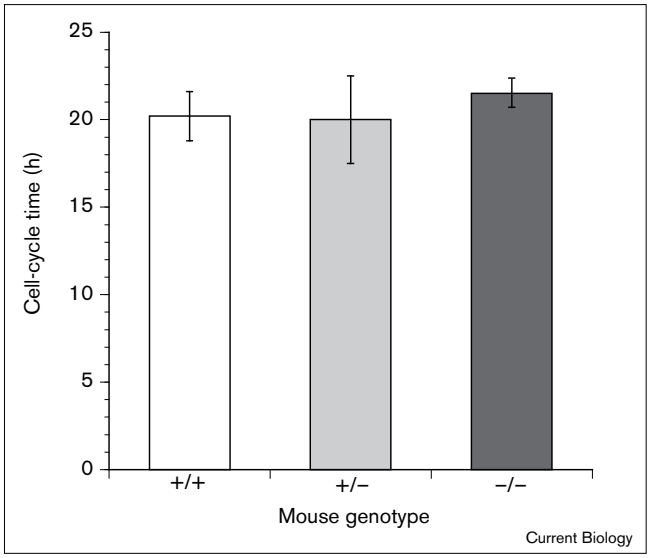
Oligodendrocyte clone sizes in P7 optic nerve cell cultures prepared from $p27^{+/+}$, $p27^{+/-}$ or $p27^{-/-}$ mice. The cells were grown at clonal density for **(a)** 5 days or **(b)** 7 days in the presence of PDGF and TH, and fresh medium and PDGF were added every 2 days. The cell number in each clone that contained mostly oligodendrocytes was recorded, and this was translated into the number of cell divisions: clones containing two cells were classified as having gone through one division, those containing three to four cells as two divisions, those containing five to eight cells as three divisions, and so on. About 100 clones were counted for each mouse, and 20 mice of each genotype were analyzed. The results are shown as means ± s.e.m. **(c)** The means for $p27^{+/+}$ and $p27^{-/-}$ cells from (b) were replotted here as curves.

mice could be explained by differences in, firstly, the cell-cycle time, secondly, the timing component of the intrinsic timer, which measures elapsed time, thirdly, the effector component of the timer, which arrests the cell cycle and initiates differentiation when the timing component indicates it is time to do so, or by some combination of these. To assess the influence of p27 on the total cell-cycle time of the precursor cells, we cultured P7 optic nerve cells from the three types of mice at clonal density in the presence of PDGF and the absence of TH to prevent their differentiation [6]. After 5 days we counted the number of cells in each oligodendrocyte precursor cell clone. In all cases, very few cells differentiated or died, the average clone size was about 32 cells, and the average calculated cell-cycle time was about 20 hours (Figure 2). Thus, p27 levels did not have an appreciable effect on the total cell-cycle time, at least in the absence of TH. To help assess the possible influence of p27 on the effector component of the intrinsic timer, we studied the effect of PDGF deprivation, which stops the cell cycle and initiates differentiation [8,9,31] by a mechanism that may or may not be the same as the one used by the normal timer that operates in the presence of PDGF.

Rate of response to PDGF deprivation

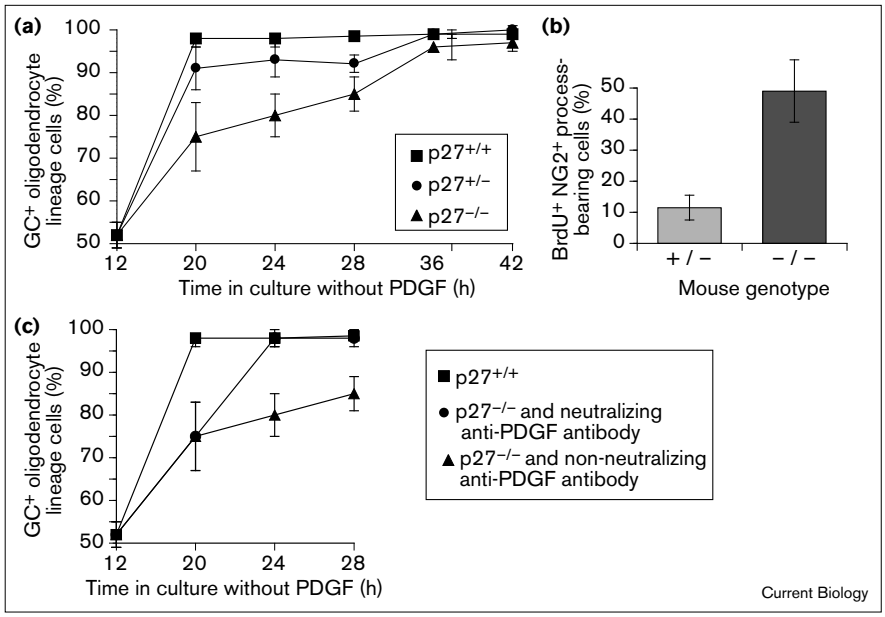
We showed previously that p27 levels increase rapidly when oligodendrocyte precursor cells are deprived of PDGF in culture — rapidly enough to play a part in arresting the cell cycle in these conditions [19]. To determine directly whether p27 contributes to stopping the cell cycle when precursor cells are deprived of PDGF, we cultured optic nerve cells from the three types of mice in the presence of TH and in the presence or absence of added PDGF. After 12, 20, 28, 36 and 44 hours we fixed the cells, immunostained them for NG2 and GC, and counted the proportion of oligodendrocyte lineage cells that was GC+

Figure 2



Average cell-cycle time of oligodendrocyte precursor cells in P7 optic nerve cultures prepared from $p27^{+/+}$, $p27^{+/-}$ and $p27^{-/-}$ mice. The cells were grown at clonal density for 5 days in the presence of PDGF and in the absence of TH. The number of cells in each clone was counted, and the average cell-cycle time was calculated from these numbers. There was little cell death, and very few cells differentiated into oligodendrocytes in these conditions. About 50–100 clones were counted for each genotype, and the results are shown as means ± s.e.m.

Figure 3



Oligodendrocyte differentiation in PDGF-deprived P7 optic nerve cell cultures. The results are shown as means ± s.e.m. **(a)** p27$^{+/+}$, p27$^{+/-}$ and p27$^{-/-}$ cells were cultured without PDGF and were fixed and immunostained for NG2 and GC after various times. The ratio of GC$^+$ oligodendrocytes to total oligodendrocyte lineage cells (GC$^+$ cells plus NG2$^+$/GC$^-$ process-bearing cells) is plotted for each time point. **(b)** Both p27$^{+/-}$ and p27$^{-/-}$ cells were grown as in (a), and after 22 h BrdU was added; after a further 22 h the cells were fixed and immunostained for NG2 and BrdU, and the proportion of NG2$^+$ process-bearing cells that were BrdU$^+$ was counted in two cultures for each genotype. **(c)** Both p27$^{+/+}$ and p27$^{-/-}$ cells were cultured as in (a), but in some cultures either neutralizing or non-neutralizing anti-PDGF antibodies were added to a final concentration of 30 μg/ml. After 20, 24 and 28 h the cells were immunostained and counted as in (a). The data for p27$^{+/+}$ precursor cells are taken from (a). In (a) and (c) about 200 cells were assessed per mouse, and 10 mice were assessed for each time point.

for each time point. For all three genotypes this proportion was about 50% at the start of the experiment and rose to about 55% when the cells were maintained in TH and PDGF for 44 hours (data not shown). As shown in Figure 3a, when p27$^{+/+}$ cells were cultured in the absence of added PDGF, more than 90% of the oligodendrocyte lineage cells were GC$^+$/NG2$^-$ oligodendrocytes by 20 hours; by contrast, many of the oligodendrocyte precursor cells in p27$^{-/-}$ cultures without PDGF differentiated more slowly, whereas those in p27$^{+/-}$ cultures differentiated at an intermediate rate. Even after 44 hours, about 5% of the p27$^{-/-}$ oligodendrocyte lineage cells were still NG2$^+$ and had the morphological features of precursor cells.

To determine whether any of the p27$^{-/-}$ precursor cells remained in cycle after 22 hours in the absence of added PDGF, we cultured P7 optic nerve cells from p27$^{+/+}$, p27$^{+/-}$ or p27$^{-/-}$ mice in the presence of TH and in the absence of PDGF, and, after 22 hours, we added bromodeoxyuridine (BrdU) to label cells synthesizing DNA. After a further 22 hours we fixed and immunostained the cells for NG2 and BrdU and counted the percentages of process-bearing NG2$^+$ cells that were BrdU$^+$. No NG2$^+$ process-bearing cells were found in cultures of p27$^{+/+}$ cells (data not shown), suggesting that all of the precursor cells had withdrawn from the cell cycle and become postmitotic oligodendrocytes. In the case of p27-deficient cells, however, some NG2$^+$ precursor cells were still present after 44 hours without PDGF, and a substantial proportion of these were BrdU$^+$ — about 10% in cultures of p27$^{+/-}$ cells and about 50% in cultures of p27$^{-/-}$ cells (Figure 3b). Most of these cycling cells were found in small groups of 2, 4 or 6 cells (data not shown), suggesting that they might have arisen from the same precursor cell.

Why did some p27-deficient precursor cells continue to divide in the absence of added PDGF? One possibility was that p27-deficient cells are especially sensitive to the small amount of endogenous PDGF made by astrocytes in the cultures. To test this possibility we used the BrdU assay to produce a dose-response curve for PDGF on wild-type P7 precursor cells and showed that, as for rat precursor cells [32], 10 ng/ml of PDGF-AA was a saturating concentration for the mouse precursor cells (data not shown). We then tested two different anti-PDGF antibodies on cultures of P7 rat and mouse optic nerve cells and showed that, while one of them completely neutralized the mitogenic effect of 10 ng/ml of PDGF-AA on oligodendrocyte precursor cells, the other one did not have a neutralizing effect (data not shown). We then cultured P7 optic nerve cells from p27$^{-/-}$ or p27$^{+/+}$ mice in the absence of PDGF and in the presence of either the neutralizing or the non-neutralizing anti-PDGF antibodies. After 12, 20 and 28 hours, we fixed and immunostained the cultures for NG2 and GC. At each time point we counted the proportion of oligodendrocyte lineage cells that was GC$^+$. As seen in Figure 3c, the p27$^{-/-}$ precursor cells differentiated into oligodendrocytes much faster in the presence of the neutralizing anti-PDGF antibodies than in the presence of the non-neutralizing anti-PDGF antibodies, suggesting

that endogenous PDGF was largely responsible for keeping the $p27^{-/-}$ cells in cycle and slowing down the rate of their differentiation. Even in the presence of the neutralizing anti-PDGF antibodies, however, it took about 4 hours longer then normal before all of the $p27^{-/-}$ cells differentiated (Figure 3c).

Discussion

The mechanisms responsible for stopping the cell cycle at the appropriate time when vertebrate precursor cells terminally differentiate are largely unknown. The stopping mechanisms are important, as they influence both the timing of differentiation and the numbers of differentiated cells generated. We previously provided evidence that the stopping mechanism in mitogen-stimulated oligodendrocyte precursor cells in culture depends on a cell-intrinsic timer [4,30], which seems to involve, in part, the accumulation of p27 [7,19]. In the present study we have analyzed the proliferative behaviour of oligodendrocyte precursor cells in clonal cultures of optic nerve cells prepared from p27-deficient mice. We show that p27 is required for the normal operation of the timer and that it also regulates the sensitivity of the cells to mitogen.

p27 levels influence when PDGF-stimulated oligodendrocyte precursors stop dividing and differentiate in culture

We showed previously that when oligodendrocyte precursor cells isolated from P7–8 rat optic nerve are cultured in the presence of PDGF and TH they undergo anywhere from zero to eight divisions before they differentiate [4,6]. We show here that when oligodendrocyte precursor cells from P7 wild-type mouse optic nerve are cultured in the same conditions they divide a maximum of only six times before they differentiate, which may be one reason why the mature mouse optic nerve contains fewer oligodendrocytes than the mature rat optic nerve. Whereas, on average, cells go through more divisions in large animals than in smaller ones, the mechanisms responsible for these differences remain a complete mystery.

The most important finding of the present study is that many oligodendrocyte precursors in clonal density cultures of optic nerve cells from p27-deficient mice undergo more cell divisions when grown in the presence of saturating amounts of PDGF and TH than do any precursors isolated from wild-type litter mates and cultured under the same conditions: whereas precursors from $p27^{-/-}$ mice can go through up to two extra divisions, precursors from $p27^{+/-}$ mice, in which p27 levels are half those found in wild-type cells, at least in some tissues ([20] and the present study), can go through only one extra division. In principle, one possible explanation for the extra divisions seen in p27-deficient precursor cells is that the cell-cycle time is shorter in these cells, so that they go through more divisions in the same period of time. This seems not to be the case, as the cell-cycle times of $p27^{-/-}$ and $p27^{+/-}$ cells are the same as wild-type cells, at least in the presence of PDGF and in the absence of TH. Thus, it seems that p27 is required for the normal operation of the cell-intrinsic timer in the precursor cells.

p27 does not act alone

Although our results indicate that p27 is required for the stopping mechanism in oligodendrocyte precursor cells to operate normally in culture, they also indicate that p27 is not required for the cells to stop dividing and differentiate in the presence of saturating amounts of PDGF. Previous studies on $p27^{-/-}$ mouse embryo fibroblasts similarly suggested that p27 was not strictly necessary for the cell-cycle arrest induced by serum deprivation [22]. It is possible that some other Cdk inhibitor, or another component of the cell-cycle control system that normally would not act in this way, takes over the function of p27 in the $p27^{-/-}$ cells, allowing the cells to stop dividing and differentiate (S. Coats, P. Whyte and J.M.R., unpublished observations). Another possibility, however, is that the stopping mechanisms in vertebrate cells normally consist of multiple components, including cell-cycle inhibitors that increase over time and cell-cycle promoters that decrease over time [1], so that if one component fails the mechanism still works, although less precisely. In this view, p27 is one of the cell-cycle inhibitors that increases over time. In *Drosophila* embryos, both an increase in the cell-cycle inhibitor Dacapo [33,34] and a decrease in the cell-cycle promoter cyclin E [35] have been shown to be required for some cells to exit the cell cycle at the appropriate time.

p27 may be part of both the timing and effector components of the cell-intrinsic timer

In principle, the failure of the cell-intrinsic timer to operate normally in p27-deficient oligodendrocyte precursor cells could reflect a defect in either the timing component that counts elapsed time, or the effector component that stops the cell cycle and initiates differentiation when the timing component indicates it is time to do so, or both. To help assess the effector component in $p27^{-/-}$ precursor cells, we have examined their response to PDGF deprivation. We find that, in the absence of added PDGF and in the presence of anti-PDGF antibodies to neutralize any endogenous PDGF made by astrocytes in the culture, $p27^{-/-}$ precursor cells stop dividing and differentiate with about a 4 hour delay compared with wild-type cells, suggesting that the effector mechanism triggered by PDGF deprivation is somewhat defective in the $p27^{-/-}$ cells. It is uncertain whether this effector mechanism is similar to the one that arrests the cycle in the presence of PDGF when the timing mechanism indicates it is time to do so. As the only known function of p27 is to inhibit cell-cycle progression, however, it seems likely that p27 also contributes to the effector component of the intrinsic timer.

A delay of a few hours in the effector component is probably insufficient to explain why p27$^{-/-}$ precursor cells divide one to two more times than wild-type cells in PDGF-stimulated clonal cultures. These results therefore suggest that the p27$^{-/-}$ cells are also defective in the timing component of the intrinsic timer. This suggestion is strongly supported by our previous findings that p27 levels progressively increase in dividing precursor cells with the expected time course for a component of the timing mechanism [19], and that p27 levels increase faster when the temperature is lowered from 37°C to 33°C, a temperature at which the timing mechanism runs faster [7]. Thus, it seems likely that p27 plays a part in both the timing and effector components of the intrinsic timer.

Cassacia-Bonnefil *et al.* [23] reported that most oligodendrocyte precursor cells isolated from cultures of neonatal p27$^{-/-}$ mouse brain failed to stop dividing and differentiate when they were deprived of mitogen in culture, whereas we find that the great majority of p27$^{-/-}$ precursor cells in optic nerve cell cultures stop dividing and differentiate within 1–2 days in these conditions. Three lines of argument suggest that the failure of most p27$^{-/-}$ precursor cells to differentiate in the mitogen-deprived cultures reported by Cassacia-Bonnefil *et al.* [23] was due to the presence of endogenous mitogen in the cultures: firstly, Cassacia-Bonnefil *et al.* were able to label 10% of wild-type cells with a 6 hour pulse of BrdU after 5 days *in vitro* without added mitogens, strongly suggesting that endogenous mitogens were present in their cultures; secondly, at the start of their cultures, 10% of the cells were astrocytes, an established source of PDGF [8–10]; and thirdly, anti-PDGF antibodies greatly increased the rate at which p27$^{-/-}$ precursor cells differentiated in our cultures, suggesting that p27$^{-/-}$ precursor cells can be kept dividing by low levels of endogenous PDGF.

As Cassacia-Bonnefil *et al.* [23] found that the number of oligodendrocytes and the amount of myelin were increased rather than decreased in the brains of their p27$^{-/-}$ mice, they suggested that factors that are present *in vivo* but not *in vitro* may induce oligodendrocyte precursor cells to exit the cell cycle and differentiate. Even in the presence of saturating amounts of PDGF, however, we find that p27$^{-/-}$ precursor cells withdraw from the cell cycle and differentiate after at most one or two extra divisions, which could readily explain the increase in oligodendrocytes and myelin in p27$^{-/-}$ mice, without having to invoke differentiation-inducing factors.

p27-deficient precursor cells are more sensitive to PDGF

Our finding that endogenous PDGF levels in optic nerve cell cultures are sufficient to keep some p27-deficient precursor cells, but not wild-type precursor cells, dividing suggests that the mutant cells are more sensitive to the low levels of PDGF found in these cultures than are wild-type cells. This finding supports previous observations that p27$^{-/-}$ cells are hypersensitive to low levels of mitogen ([20,36] and see below). There are a number of observations that connect p27 to mitogen responsiveness. When p27 levels are artificially elevated in various cell lines by transfection, the cells become unresponsive to mitogens and arrest in G1 (reviewed in [15]). Conversely, when the expression of p27 in 3T3 cells is inhibited with antisense oligonucleotides, the cells continue to divide following mitogen deprivation [36]. Moreover, p27$^{-/-}$ thymocytes seem to be more sensitive to the mitogenic effect of interleukin-2 than are wild-type thymocytes [20]. The p27 protein counteracts the effects of mitogens mainly by inhibiting the activity of cyclin–Cdk2 complexes, which are required for progression through G1 and entry into S phase (reviewed in [15]). Conversely, mitogens can counteract the effect of p27 by activating the transcription of *c-myc*, which can in turn counteract p27 action [37,38]. Mitogens can also increase p27 degradation [39–42] and decrease p27 synthesis [43]. Thus, there are probably multiple reasons why p27-deficient oligodendrocyte precursor cells are more sensitive to the mitogenic effect of PDGF than wild-type cells.

In principle, the phenotype of p27-deficient mice could be explained entirely by the increased mitogen sensitivity of various cell types. Our results with p27-deficient oligodendrocyte precursor cells, however, cannot easily be explained in this way, as these cells undergo more divisions than wild-type cells even in the presence of saturating amounts of PDGF, suggesting that the mutant cells have an intrinsic defect in their stopping mechanism, as discussed above.

Our suggestion that defects in cell-intrinsic timers may explain at least part of the phenotype of p27-deficient mice assumes that such timers work *in vivo*, as well as *in vitro*. Two lines of indirect evidence suggest that the intrinsic timer in an oligodendrocyte precursor cell operates *in vivo* to determine when the cell stops dividing and differentiates. First, whereas the delivery of extra PDGF to P8 rats greatly inhibited the normal cell death of newly formed oligodendrocytes in the optic nerve, it did not increase the proliferation of oligodendrocyte precursor cells in the nerve [5], suggesting that the precursors do not normally stop dividing and start to differentiate because of PDGF deprivation, at least at this stage of development. Second, a cell-lineage study in the developing rat brain using retrovirus-mediated gene transfer suggests that the progeny of individual oligodendrocyte precursor cells tend to differentiate more or less synchronously, as clones of oligodendrocyte lineage cells contained either precursors or oligodendrocytes, but not both [44]; this finding is consistent with the possibility that oligodendrocyte differentiation *in vivo* is controlled largely by an intrinsic timer rather than by mitogen deprivation or differentiation-inducing factors.

p27 may help many types of developing vertebrate cells to exit the cell cycle

It is likely that p27 plays a part in stopping cell division in many cell lineages during vertebrate development. Levels of p27 are high in many tissues where cells are exiting the cell cycle and terminally differentiating [45–47]. Most importantly, in $p27^{-/-}$ mice, all organs that have been examined are larger than normal as a result of an increase in cell number, apparently due to an increase in cell proliferation rather than a decrease in cell death [20–22]. Thus, our findings may be relevant to many vertebrate cell lineages where precursors divide a limited number of times before they terminally differentiate.

Cdk inhibitors also play a part in stopping cell division in developing invertebrates. The *Drosophila dacapo* gene, for example, encodes a p27-like Cdk inhibitor, which is expressed in a number of developing tissues, including the epidermis, just as cells exit the cell cycle. In *dacapo* mutants, epidermal precursor cells go through an extra cell division before differentiating [33,34]. Thus some of the mechanisms involved in cell-cycle exit may be widely conserved in animals. The *dacapo* gene, however, is controlled transcriptionally and is expressed only transiently at high levels when cells exit the cell cycle [33,34], whereas p27 protein progressively accumulates as oligodendrocyte precursor cells proliferate and remains high in terminally differentiated oligodendrocytes [19], as well as in other postmitotic cells [15,16]. Moreover, p27 levels seem to be regulated mainly post-transcriptionally [48,49], by protein sequestration [48], translational control [39] or ubiquitin-dependent proteolysis [50].

In summary, our findings suggest that an increase in p27 is part of the mechanism that stops precursor cell division at the appropriate time in a number of vertebrate cell lineages. The other components of the stopping mechanism(s) remain to be discovered, but it seems likely that they will include both intracellular cell-cycle promoters and intracellular cell-cycle inhibitors, which decrease and increase, respectively, to help arrest the cell cycle at the correct time.

Materials and methods

Optic nerve cultures

The p27-deficient mice were produced as previously described [20] and bred in the animal facility at University College London. All chemicals were from Sigma unless indicated otherwise. Optic nerves were removed from P7 mice and dissociated as previously described [19]. Briefly, the nerves from individual mice were cut into fragments, treated with trypsin (0.05%, Boehringer Mannheim) in Earle's balanced salt solution (EBSS), and dissociated with a 200 µl micropipette (Gilson) in Dulbecco's modified Eagle's medium (DMEM) containing 30% (volume : volume) fetal calf serum (FCS) and 0.04% DNase. The cells were plated onto poly-D-lysine (PDL)-coated glass coverslips (3,000 cells per coverslip) or into a Nunc tissue culture flask (3,000 cells per flask), and grown in 8% CO_2 at 37°C in Bottenstein-Sato medium [51], modified as previously described [52]. Forskolin and PDGF-AA (Peprotech) were added to a final concentration of 15 µg/ml and 10 ng/ml, respectively. In some cases, TH was added as triiodothyronine and thyroxine at 40 ng/ml each. Where indicated, the medium was supplemented with either 50% (volume : volume) astrocyte-conditioned medium prepared from newborn rat brain astrocytes, which were prepared and grown for 3 days in DMEM as previously described [53], or 5% (volume : volume) Müller-cell-conditioned medium prepared from newborn mouse retinal Müller cells, which were prepared and grown for 2 days in DMEM as previously described [54]. All of the factors were used at concentrations that were shown to be on the plateau of their dose response curves. Half of the medium and additives were changed every 2 days. In some experiments, optic nerve cell cultures, growing in the absence of PDGF, were treated with either neutralizing rabbit anti-human PDGF-AA antibodies (R&D systems; AB-221-NA) or non-neutralizing rabbit anti-human PDGF-AA antibodies (Biogenesis), which were added to the medium at the beginning of the culture period at a concentration of 30 µg/ml. We found that the neutralizing antibodies at this concentration abolished the mitogenic activity of 10 ng/ml of PDGF-AA on oligodendrocyte precursor cells in both rat and mouse optic nerve cell culture, whereas the non-neutralizing antibodies had no effect.

Immunofluorescence staining

For staining cells on their surface for NG2 or GC, cells were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer for 5 min at room temperature. After washing, the cells were incubated for 15 min in 50% normal goat serum (NGS) to block nonspecific staining. They were then incubated in either a rabbit anti-NG2 antiserum [29] (diluted 1:100), followed by biotinylated-anti-rabbit IgG antibodies (Amersham; diluted 1:100) and Texas-red-coupled streptavidin (Amersham; diluted 1:100) or monoclonal anti-GC antibody [26] (ascites fluid diluted 1:100) followed by FITC-coupled goat anti-mouse IgG3 antibodies (Nordic; diluted 1:100). For BrdU staining, BrdU (Boehringer Mannheim) was added to the culture medium to a final concentration of 10 µM. Cells were fixed in 100% methanol at –20°C for 5 min and then incubated in 2 N HCl for 10 min to denature the DNA, followed by 0.1 M sodium borate pH 8.5 for 10 min. The cells were then incubated in 50% NGS for 15 min, then monoclonal anti-BrdU antibody [55] (supernatant diluted 1:1), and then fluorescein-coupled goat anti-mouse IgG1 antibodies (Amersham; diluted 1:100). All incubations were for 25 min at room temperature, and the dilutions were in Tris-buffered saline containing 1% bovine serum albumin and 10 mM L-lysine. The coverslips were mounted in Citifluor mounting medium (Citifluor UKC) on glass slides, sealed with nail varnish, and then examined with a Zeiss Universal fluorescence microscope.

Western blotting

The genotype of each mouse was determined by western blotting of a brain extract using affinity-purified rabbit anti-p27 antibodies (Santa-Cruz) and the ECL method (Amersham) according to the manufacturer's instructions. Half of the brain was homogenised with a pestle in 500 µl sample buffer consisting of 125 mM Tris pH 6.95, 15% sucrose, 4% SDS, 10 mM EDTA, 0.0001% bromophenol blue, and 100 mM dithiothreitol (added fresh) and boiled for 5 min. The DNA was sheared by ultrasonication for 20 sec, and the extracts were stored at –20°C until use. Protein concentration was assayed by Coomassie staining (Pierce). Samples (10 µl) were boiled briefly and analyzed by SDS–PAGE, using a 12% gel (mini-protean, Biorad), prepared using a modified Laemmli gel recipe. The proteins were transferred from the gel to nitrocellulose, which was stained with india ink to check for the quality of the transfer and that an equal amount of protein was loaded in each lane. The nitrocellulose was then blocked in phosphate-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk (PBST-M), incubated in the affinity-purified rabbit anti-p27 antibodies (diluted 1:1000 in PBST-M), followed by biotinylated donkey anti-rabbit Ig antibodies (Amersham; diluted 1:3000 in PBST-M) and then streptavidin coupled to horseradish peroxidase (Amersham; diluted 1:3000 in PBST-M with 0.5% caseine). The labeled proteins were detected by the ECL method (Amersham). The genotype of each mouse was confirmed independently by western blotting of heart extracts (Robert Poolman, personal communication).

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References

1. Raff M: **Size control: the regulation of cell numbers in animal development.** *Cell* 1996, **86:**173-175.
2. Barres B, Raff M: **Control of oligodendrocyte number in the developing rat optic nerve.** *Neuron* 1994, **12:**935-942.
3. Small R, Riddle P, Noble M: **Evidence for migration of oligodendrocyte-type-2 astrocyte progenitor cells into the developing rat optic nerve.** *Nature* 1987, **328:**155-157.
4. Temple S, Raff M: **Clonal analysis of oligodendrocyte development in culture: evidence for a developmental clock that counts cell divisions.** *Cell* 1986, **44:**773-779.
5. Barres B, Hart I, Coles H, Burne J, Voyvodic J, Richardson W, Raff M: **Cell death and control of cell survival in the oligodendrocyte lineage.** *Cell* 1992, **70:**31-46.
6. Barres B, Lazar M, Raff M: **A novel role for thyroid hormone, glucocorticoids and retinoic acid in timing oligodendrocyte development.** *Development* 1994, **120:**1097-1108.
7. Gao F-B, Durand B, Raff M: **Oligodendrocyte precursor cells count time but not cell divisions before differentiation.** *Curr Biol* 1997, **7:**152-155.
8. Richardson WD, Pringle N, Mosley MJ, Westermark B, Dubois-Dalcq M: **A role for platelet-derived growth factor in normal gliogenesis in the central nervous system.** *Cell* 1988, **53:**309-319.
9. Noble M, Murray K: **Purified astrocytes promote the *in vitro* division of a bipotential glial progenitor cell.** *EMBO J* 1984, **3:**2243-2247.
10. Raff M: **Glial cell diversification in the rat optic nerve.** *Science* 1989, **243:**1450-1455.
11. Bögler O, Noble M: **Measurement of time in oligodendrocyte-type 2 astrocyte (O-2A) progenitors is a cellular process distinct from differentiation or division.** *Dev Biol* 1994, **162:**525-538.
12. Lees E: **Cyclin dependent kinase regulation.** *Curr Opin Cell Biol* 1995, **7:**773-780.
13. Morgan D: **Principles of CDK regulation.** *Nature* 1995, **374:**131-134.
14. Sherr C: **G1 phase progression: cycling on cue.** *Cell* 1994, **79:**551-556.
15. Sherr C, Roberts J: **Inhibitors of mammalian G1 cyclin dependent kinases.** *Genes Dev* 1995, **9:**1149-1163.
16. Harper W, Elledge S: **Cdk inhibitors in development and cancer.** *Curr Opin Genet Dev* 1996, **6:**56-64.
17. Matsuoka S, Edwards M, Bai C, Parker S, Zhang P, Baldini A, *et al.*: **p57 Kip2, a structurally distinct member of the p21 Cip1 inhibitor family, is a candidate tumor suppressor gene.** *Genes Dev* 1995, **9:**650-662.
18. Parker S, Eichele G, Zhang P, Rawls A, Sands A, Bradley A, *et al.*: **p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells.** *Science* 1995, **267:**1024-1027.
19. Durand B, Gao F-B, Raff M: **Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation.** *EMBO J* 1997, **16:**306-317.
20. Fero M, Rivkin M, Tasch M, Porter P, Carow C, Firpo E, *et al.*: **A syndrome of multi-organ hyperplasia with features of gigantism, tumorigenesis and female sterility in p27Kip1-deficient mice.** Cell 1996, **85:**733-744.
21. Kiyokawa H, Kineman R, Manova-Todorova K, Soares V, Hollman E, Ono M, *et al.*: **Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27Kip1.** *Cell* 1996, **85:**721-732.
22. Nakayama K, Ishida N, Shirane M, Inomata A, Inoue T, Shishido N, *et al.*: **Mice lacking p27Kip1 display increased body size, multiple organ hyperplasia, retinal dysplasia and pituary tumors.** *Cell* 1996, **85:**707-720.
23. Casaccia-Bonnefil P, Tikoo R, Kiyokawa H, Friedrich VJ, Chao MV, Koff A: **Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27/Kip1.** *Genes Dev* 1997, **11:**2335-2346.
24. Raff MC, Mirsky R, Fields KL, Lisak RP, Dorfman SH, Silberberg DH, *et al.*: **Galactocerebroside is a specific cell-surface antigenic marker for oligodendrocytes in culture.** *Nature* 1978, **274:**813-816.
25. Raff MC, Miller RH, Noble M: **A glial progenitor cell that develops *in vitro* into an astrocyte or an oligodendrocyte depending on culture medium.** *Nature* 1983, **303:**390-396.
26. Ranscht B, Clapshaw P, Price J, Noble M, Seifert W: **Development of oligodendrocytes and schwann cells studied with a monoclonal antibody against galactocerebroside.** *Proc Natl Acad Sci USA* 1982, **79:**2709-2713.
27. Eisenbarth GS, Walsh FS, Nirenberg M: **Monoclonal antibody to a plasma membrane antigen of neurons.** *Proc Natl Acad Sci USA* 1979, **76:**4913-4917.
28. Fanarraga M, Sommer I, Griffiths I: **O-2A progenitors of the mouse optic nerve exhibit a developmental pattern of antigen expression different from the rat.** *Glia* 1995, **15:**95-104.
29. Stallcup W, Beasley L: **Potential glial precursor cells of the optic nerve express the NG2 proteoglycan.** *J Neurosci* 1987, **7:**2737-2744.
30. Gao F-B, Apperly J, Raff M: **Cell size control and a cell-intrinsic maturation program in proliferating oligodendrocyte precursor cells.** *J Cell Biol* 1997, **138:**1367-1377.
31. Raff M, Lillien L, Richardson W, Burnem J, Noble M: **Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture.** *Nature* 1988, **333:**562-565.
32. Barres B, Schmid R, Sendtner M, Raff M: **Multiple extracellular signals are required for long term oligodendrocyte survival.** *Development* 1993, **118:**283-295.
33. Lane M, Sauer K, Wallace K, Jan Y-N, Lehner C, Vaessin H: **Dacapo, a cyclin-dependent kinase inhibitor stops cell proliferation during *Drosophila* development.** *Cell* 1996, **87:**1225-1235.
34. de Nooij J, Letendre M, Hariharan I: **A cyclin-dependent kinase inhibitor, Dacapo, is necessary for timely exit from the cell cycle during *Drosophila* embryogenesis.** *Cell* 1996, **87:**1237-1247.
35. Knoblich J, Sauer K, Jones L, Richardson H, Saint R, Lehner C: **Cyclin E controls S phase progression and its down regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation.** *Cell* 1994, **77:**107-120.
36. Coats S, Flanagan W, Nourse J, Roberts J: **Requirement of p27/Kip1 for restriction point control of the fibroblast cell cycle.** *Science* 1996, **272:**877-880.
37. Perez-Roger I, Solomon DL, Sewing A, Land H: **Myc activation of cyclinE/Cdk2 kinase involves induction of cyclin E gene transcription and inhibition of p27(Kip1) binding to newly formed complexes.** *Oncogene* 1997, **14:**2373-2381.
38. Vlach J, Hennecke S, Alevizopoulos K, Conti D, Amati B: **Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc.** *EMBO J* 1996, **15:**6595-6604.
39. Hengst L, Reed S: **Translational control of p27Kip1 accumulation during the cell cycle.** *Science* 1996, **271:**1861-1864.
40. Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE: **Cyclin E–CDK2 is a regulator of p27Kip1.** *Genes Dev* 1997, **11:**1464-1478.
41. Winston J, Dong F, Pledger WJ: **Differential modulation of G1 cyclins and the Cdk inhibitor p27kip1 by platelet-derived growth factor and plasma factors in density-arrested fibroblasts.** *J Biol Chem* 1996, **271:**11253-11260.
42. Leone G, DeGregori J, Sears R, Jakoi L, Nevins JR: **Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F.** *Nature* 1997, **387:**422-426.
43. Millard SS, Yan JS, Nguyen H, Pagano M, Kiyokawa H, Koff A: **Enhanced ribosomal association of p27(Kip1) mRNA is a mechanism contributing to accumulation during growth arrest.** *J Biol Chem* 1997, **272:**7093-7098.
44. Grove EA, Williams BP, Li DQ, Hajihosseimi M, Friedrich A, Price J: **Multiple restricted lineages in the embryonic rat cerebral cortex.** *Development* 1993, **117:**553-561.
45. Firpo E, Koff A, Solomon M, Roberts J: **Inactivation of a cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes.** *Mol Cell Biol* 1994, **14:**4889-4901.
46. Kato J-Y, Matsuoka M, Polyak K, Massagué J, Sherr C: **Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27 Kip1) of cyclin-dependent kinase 4 activation.** *Cell* 1994, **79:**487-496.
47. Nourse J, Firpo E, Flanagan W, Coats S, Polyak K, Lee M, *et al.*: **Interleukin-2-mediated elimination of the p27Kip1 cyclin dependent kinase inhibitor prevented by rapamycin.** *Nature* 1994, **372:**570-573.
48. Polyak K, Lee M-H, Erdjument-Bromage H, Koff A, Roberts J, Tempst P, Massagué J: **Cloning of p27 Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals.** *Cell* 1994, **78:**59-66.

49. Toyoshima H, Hunter T: **p27, a novel inhibitor of G1 cyclin-cdk protein kinase activity, is related to p21.** *Cell* 1994, **78:**67-74.
50. Pagano M, Tam S, Theodoras A, Beer-Romero P, Del Sal G, Chau V, *et al.*: **Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27.** *Science* 1995, **269:**682-685.
51. Bottenstein J, Sato G: **Growth of a rat neuroblastoma cell line in serum-free supplemented medium.** *Proc Natl Acad Sci USA* 1979, **76:**514-517.
52. Lillien L, Raff M: **Differentiation signals in the CNS: type-2 astrocyte development *in vitro* as a model system.** *Neuron* 1990, **5:**111-119.
53. Lillien LE, Sendtner M, Rohrer H, Hughes SM, Raff MC: **Type-2 astrocyte development in rat brain cultures is initiated by a CNTF-like protein produced by type-1 astrocytes.** *Neuron* 1988, **1:**485-494.
54. Neophytou C, Vernallis AB, Smith A, Raff MC: **Müller-cell-derived leukaemia inhibitory factor arrests rod photoreceptor differentiation at a postmitotic pre-rodstage of development.** *Development* 1997, **124:**2345-2354.
55. Magaud J, Sargent I, Mason D: **Detection of human white cell proliferative responses by immunoenzymatic measurement of bromodeoxyuridine uptake.** *J Immunol Methods* 1988, **106:**95-100.

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